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DIFFERENTIATED MICRODOMAINS ON THE LUMINAL SURFACE OF THE ENDOTHELIA OF MURINE DIAPHRAGM MICROVESSELS

FRED E. SANFORD

1985

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DIFFERENTIATED MICRODOMAINS ON THE LUMINAL SURFACE OF THE ENDOTHELIA OF MURINE DIAPHRAGM MICROVESSELS

Fred E. Santoro

A Thesis Submitted to the
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Requirements for the Degree of
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DEDICATION

To the people of The Institute of Cellular Biology and Pathology, Bucharest, Romania, for their kindness, generosity and hospitality. I will always be grateful for having been the first American to undertake a research project at this outstanding institution.

Dedic această teză oamenilor din Institutul de Biologie şi Patologie Celulară, Bucuresţi, România, pentru amabilitatea, generozitatea şi ospitalitatea lor. Voi fi întotoleauna recunoscător pentru faptul că am fost primul American care să efectuieze un proiect de cercetare în aceasta prestigioasă instituţie.

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I am also very grateful to the people of The Institute of Cellular Biology and Pathology for their help and encouragement during my three months with them. Dr. Nicolae Simionescu, the director of the Institute, was always very supportive. I also appreciate the help of S. Tancov, M. Misici, M. Mitroaica, V. Ionescu, H. Stukenbrok, M. Haire, P. Male, P. Ossario, C. Davis and M.L. Wootton.

I would like to give special thanks to Dr. Maya Simionescu, who acted as my thesis advisor. She was very enthusiastic when I first approached her with the thought of working with her in Bucharest, Romania, and continued to be a supportive teacher and concerned advisor throughout the time we have worked together.

ABSTRACT

The existence of luminal endothelial microdomains, formed by a preferential distribution of anionic sites, has been recently described for certain murine tissue microvessels (jejunum, pancreas, lung and liver). It has been proposed that structural elements of the endothelium devoid of negative charge (plasmalemmal vesicles in particular) may facilitate the transport of plasma proteins across the endothelial cell. In this study, the anionic sites of the luminal aspect of the continuous endothelium of murine diaphragm microvessels were investigated. Cationized ferritin (pI 8.4) was used to probe the anionic sites of these microvessels through the use of in situ perfusion techniques. Labeling was analyzed with the aid of the electron microscope and quantified by morphometric analysis of the micrographs obtained. It was found that the plasmalemma proper was labeled by cationized ferritin, albeit in a discontinuous manner, whereas the majority of plasmalemmal vesicles and their stomatal diaphragms, when present, were not labeled. A small difference in the percentage of plasmalemmal labeling was found between the different segments of the microvasculature. Specifically, a higher percentage of the plasmalemma of arteriolar and venular endothelial cells was labeled with cationized ferritin when compared to the labeling of capillary endothelial cells. It was also found that the luminal plasmalemma of arteriolar and venular endothelial cells of old mouse microvessels was less extensively labeled than the luminal plasmalemma of endothelial cells from the respective microvessels of young animals. This decreased cell surface negativity associated with aging may be a contributing factor in the pathogenesis of atherosclerosis and thrombosis.

INTRODUCTION

During the past few decades, much has been learned about the structure and function of the endothelium, the specialized epithelial cells of the microvasculature. Recent experiments have demonstrated that there is a preferential distribution of anionic (negative) sites and lectin binding sites on the luminal surface of the endothelium. Several theories have been advanced to define the role of negative surface charge on cells in general. In the case of vascular endothelium, specific functions relating to anti-thrombogenesis and vascular permeability have been attributed to the negative charge distribution on the endothelial luminal surface.

Cationized ferritin (CF) has been used in conjunction with electron microscopy to elucidate the surface charge characteristics of endothelial cells, especially on fenestrated endothelium (N. Simionescu and M. Simionescu, 1978; N. Simionescu et al., 1981) and discontinuous endothelium (Ghitescu and Fixman, 1984). There are few reports in the literature where this technique has been utilized to investigate continuous endothelium, the most commonly encountered endothelium in mammals, humans included.

In this study, the bipolar microvascular fields of mouse diaphragm were probed with cationized ferritin to determine the fine distribution of anionic sites on the luminal aspect of the continuous endothelium in sequential microvascular segments (arterioles, capillaries, venules). Selected microvessels of young and old mice were examined with the use of an electron microscope and the perfused cationized ferritin binding pattern was characterized. The goals of this investigation were to compare the distribution of anionic sites found on the luminal surface

of mouse diaphragm (continuous) endothelium to that already described for other endothelia, to determine if segmental variation existed in this anionic site distribution, and to see if a difference in cationized ferritin binding was present between young and old animals.

Preliminary results of this study have been presented elsewhere (M. Simionescu et al., 1983).

ENDOTHELIUM OF THE MICROVASCULATURE

The microscopic segment of the circulatory system known as the microvasculature is composed of arterioles, capillaries and venules. The endothelium of these microvessels is a specialized and highly differentiated epithelium with a permeability to water and solutes that is two to three orders of magnitude higher than that found in any other epithelium (Renkin, 1977). The electron microscope has been an important tool in the study of the structure and function of these remarkable cells.

ULTRASTRUCTURE

The blood-endothelial interface is formed by the glycocalyx (cell coat) of the luminal plasmalemma of the endothelial cells, and a layer of adsorbed plasma proteins. The glycocalyx is resistant to removal by washing of the vasculature, and is composed of sialoconjugates, oligosaccharide moieties of plasmalemmal glycoproteins and glycolipids, and proteoglycans (M. Simionescu et al., 1981; M. Simionescu et al., 1982a; N. Simionescu, 1983; N. Simionescu and M. Simionescu, 1983). The adsorbed plasma proteins, which include α_2-macroglobulin, fibrin[ogen], heparin, lipoprotein lipase and albumin, can be removed by vasculature washing. Together, these two components form a diffusion barrier of 10 to 30 nm in thickness (N. Simionescu and M. Simionescu, 1983).

The capillary endothelial cell typically has a thickened zone around the nucleus called the perikaryon and an attenuated segment (Clementi and Palade, 1969). In the attenuated segment, the distance between the luminal and abluminal aspects of the cell is reduced to 0.2 to 0.4 μm (N. Simionescu, 1981). Another structural characteristic of

endothelial cells is their high density of plasmalemmal vesicles, which was first described by Palade (Palade, 1953). These vesicles have an outer diameter of 50 to 90 nm (average of 70 nm)(Palade et al., 1979) and apparently exist in three locations: vesicles open to the blood front, vesicles open to the tissue front, and vesicles enclosed within the cytoplasm (Bruns and Palade, 1968a). Vesicles opening to the tissue and blood fronts are often spanned by a single-layered diaphragm which is devoid of lipids. The density of plasmalemmal vesicles varies among tissues and along the different segments of the microvasculature within a given tissue. For example, the density of plasmalemmal vesicles is ten times higher in myocardial capillaries than in brain capillaries (N. Simionescu, 1983) and is significantly higher in the capillaries and venules than in the arterioles of mouse diaphragm (N. Simionescu et al., 1978b).

It is believed that plasmalemmal vesicles may give rise to two other endothelial structures, the transendothelial channels and the fenestrae (N. Simionescu et al., 1975; Palade et al., 1979). Transendothelial channels, which are composed of a single vesicle or two or more fused vesicles, are water filled passages which connect the luminal and abluminal aspects of the endothelial cell and are approximately 70 nm in diameter in their largest transverse dimension (N. Simionescu et al., 1975; Palade et al., 1979). The channels often have strictures, which narrow their diameter to approximately 10 nm at their openings at the tissue or blood fronts or at the fusion site of the forming plasmalemmal vesicles from which they are derived (N. Simionescu et al., 1975). Fenestrae are transcellular openings, with a diameter of 50 to 80 nm which are usually closed by a single layered diaphragm (M. Simionescu et al., 1974).

Other structural elements of the endothelium include the coated pits and coated vesicles and the intercellular junctions. Coated vesicles are somewhat larger and much less frequently observed than plasmalemmal vesicles (Palade et al., 1979). Both the occluding (tight) and communicating (gap) type junctions are seen between endothelial cells (N. Simionescu, 1983). Arteriolar endothelium contains both occluding and communicating junctions, whereas capillary endothelium has occluding junctions only. Communicating junctions also do not occur between the endothelial cells of postcapillary venules, which are characterized by loosely organized lines of fusion (M. Simionescu et al., 1975). A summary of the structural elements of the endothelial cells is given in Figure 1.

There are three subtypes of capillaries: fenestrated, continuous and discontinuous, which are characterized by the specific structural elements present or absent in their respective endothelia. Fenestrated capillaries, which are found in the mucosa of the gastrointestinal tract, endocrine glands, the renal glomerulus and peritubular vascular network, the choroid plexus and the ciliary body, have many fenestrae in addition to all the other structural elements discussed. The fenestrated capillaries of the renal glomerulus are further characterized by larger fenestrae, typically devoid of diaphragms, and a thicker basal lamina or basement membrane. Continuous capillaries which occur in the brain, muscle tissue, the lung and many other tissues, have all the same structural elements except fenestrae. The endothelium of both of these capillary subtypes are associated with a continuous basal lamina. Discontinuous capillaries, also called sinusoids, are characterized by large transcellular discontinuities of up to hundreds

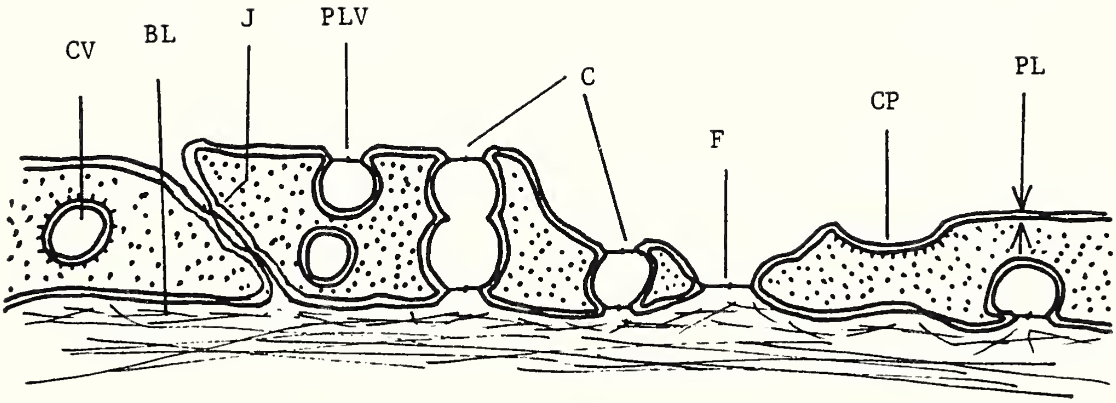

FIGURE 1 Schematic representation of the stuctural elements of the endothelial cell. CV: coated vesicle, BL: basal lamina, J: intercellular junction, PLV: plasmalemmal vesicle, C: transendothelial channel, F: fenestra, CP: coated pit, PL: plasmalemma proper.

of nanometers. The basal lamina of these capillaries is either discontinuous or absent. This type of capillary is found in the liver, spleen and parts of the bone marrow.

TRANSPORT

The study of capillary transport during the past two decades has focussed on the role of the structural elements of continuous and fenestrated endothelium in the transport of macromolecules across the capillary wall. Experiments using tracers of different sizes have been employed in an attempt to characterized transport in terms of the pore theory (Pappenheimer et al., 1951) of capillary permeability. This theory stipulates that capillary transport occurs through two populations of "pores" within the endothelium: small pores with a diameter of 6 to 9 nm and large pores with a diameter of 50 to 70 nm.

In early tracer experiments, horseradish peroxidase (molecular

diameter: 5 to 6 nm) was used to investigate the permeability of continuous endothelium. In one study (Karnovsky, 1967) it was reported that this molecule traversed the capillary wall through intercellular junctions. More recent experiments with this tracer, however, have failed to demonstrate the movement of horseradish peroxidase through the intercellular junctions, and have implicated plasmalemmal vesicles in the transport of this molecule from the blood to the tissue front (Boyles et al., 1981). This vesicular shuttling of molecules across the endothelial cell by the efficient coupling of endocytosis and exocytosis has come to be known as transcytosis (N. Simionescu, 1981). In fenestrated capillaries, horseradish peroxidase has been shown to traverse the capillary wall via diaphragmed fenestrae and to a lesser extent by plasmalemmal vesicles, but not via intercellular junctions (Clementi and Palade, 1969).

Even smaller tracers have been used in the study of capillary permeability. Whale skeletal muscle myoglobin (dimensions: 2.5 X 3.4 X 4.2 nm) was shown to cross continuous endothelium by transcytosis, but not through intercellular junctions (N. Simionescu et al., 1973). Heme-undecapeptide and heme-octapeptide (approximate diameter: 2 nm) were transported from the lumen to the pericapillary space of continuous capillaries by transcytosis through plasmalemmal vesicles and transendothelial channels (N. Simionescu et al., 1975; N. Simionescu et al., 1978b). Intercellular junctions were impermeable to these tracers in all segments of the microvasculature except for a fraction of junctions which seem to be open at the level of pericytic venules (N. Simionescu et al., 1973b). Microperoxidase (molecular diameter: 2 nm), however, was shown to cross the continuous endothelium of mouse

diaphragm through some intercellular junctions (Wissig and Williams, 1978). In these experiments, plasmalemmal vesicles and transendothelial channels did not appear to be significantly involved in the transport of this tracer.

Ferritin, a larger molecule with a diameter of approximately 11 nm, was transported across fenestrated capillaries by plasmalemmal vesicles and through certain fenestrae, but did not pass through intercellular junctions (Clementi and Palade, 1969). This molecule was also shown to be transported across continuous endothelium by plasmalemmal vesicles (Bruns and Palade, 1968b). In vitro experiments upon capillary isolates from rat epididymal fat demonstrated plasmalemmal vesicle mediated endocytosis followed by exocytosis of fluorescently labeled ferritin (Williams and Wagner, 1979). In the continuous capillaries of frog mesentery, plasmalemmal vesicles were also shown to be involved in transendothelial transport or ferritin, but the mechanism of transport was believed to be that of fusion and mixing of vesicular contents (Clough and Michel, 1981).

It should be noted that some investigators believe that plasmalemmal vesicles may not be involved in microvessel transport. Bundgaard et al. observed that vesicles which were considered to be enclosed within the endothelial cytoplasm of fixed frog mesenteric capillaries were stained by perfused tannic acid (Bundgaard et al., 1979). They concluded that these "free" cytoplasmic vesicles were continuous with membrane invaginations and therefore actually open to the lumen. Serial 15 nm sections through frog mesenteric capillaries were examined, and it was found that less than one percent of plasmalemmal vesicles were free within the cytoplasm (Frøkjaer-Jensen, 1980). Rat heart capillaries were also studied, and this three-

dimensional reconstruction of serial thin sections did not show free vesicles within the endothelial cytoplasm (Bundgaard et. al., 1983). The authors point out, however, that they can not rule out the possibility that these findings were due to a fixation artifact.

The effect of the charge of a molecule on its transport across the capillary wall has been studied. It has been demonstrated that rabbit ear capillaries are more permeable to neutral dextrans than to negatively charged dextrans of the same size (Garby and Areekul, 1970). Many studies have shown that glomerular capillaries have a decreased permeability to anionic macromolecules when compared to neutral molecules of similar size (Chang et al., 1975; Brenner et al., 1978); in such cases of charge selectivity, the glomerular basement membrane has been implicated as the main barrier (Farquhar, 1978).

Chemically-specific transport has also been demonstrated. Microvessel isolates from rat epididymal fat showed uptake of ferritin, but not albumin (Wagner et al., 1980). This selectivity was not found to be related to molecular size (ferritin is larger than albumin) or net charge (both proteins have a similar net charge). Glycosylated albumin (Williams et al., 1981), myoglobin and ovalbumin (Williams and Solenski, 1984) were more avidly taken up by isolated rat epididymal endothelial cells than were the respective non-glycosylated proteins. Recent experiments have also shown receptor mediated endocytosis, in addition to low affinity transcytosis, of LDL in mouse aorta (Vasile et al., 1983).

Thus, microvascular permeability has been found to be a function of the size, electrical charge and biochemical nature of the transported molecule. It has been postulated that in visceral capillaries fenestrae

without diaphragms or with compliant diaphragms and plasmalemmal vesicles appear to be the structural equivalents of the large pores, and the fenestral diaphragms seem to serve as the small pores (N. Simionescu et al., 1976; Palade et al., 1979). In continuous capillaries, plasmalemmal vesicles serve as the structural equivalent of both the small and large pores, and affect transport either through dissipative transcytosis, fusion to form transendothelial channels, or both (N. Simionescu et al., 1976; Palade et al., 1979; Wagner and Casley-Smith, 1981). As noted above, however, some question the role of plasmalemmal vesicles in endothelial permeability. There is also conflicting evidence in the literature concerning the role of intercellular junctions in the transport of small molecules. Some investigators consider these junctions to be the structural equivalent of the small pores (Wissig and Williams, 1978) whereas others believe that intercellular junctions do not play a role in the transport of molecules with a diameter greater than two nanometers except at the level of pericytic venules (N. Simionescu et al., 1976; Palade et al., 1979).

BIOCHEMISTRY

Relatively little is known about the biochemistry of the endothelium (Palade, 1982); much of what is known has been learned during the past fifteen years. Many biosynthetic products of endothelial cells have been discovered. Specific enzymes and receptors have also been found on or in these cells.

Heparan sulfate proteoglycans are synthesized and secreted by rabbit endothelial cells in culture (Buonassisi and Colburn, 1982) and cultured calf aorta endothelia synthesize and secrete fibronectin (Macarak et al., 1978) and type IV collagen (Howard et al., 1976).

Endothelial cells cultured from human umbilical vein have been found to make major blood group antigens (Jaffe et al., 1973b).

Endothelial cells also produce coagulant and anticoagulant substances. Cultured human endothelial cells were found to secrete and to incorporate into their plasmalemma a factor VIII antigen (Jaffe et al., 1973a). This antigen was subsequently identified as factor VIII:vWF, the major component of factor VIII complex (Jaffe, 1982). Plasminogen inhibitor is synthesized by cultured endothlelial cells (Loskutoff and Edgington, 1977) and tissue factor (thromboplastin) has been detected on endothelial cell surfaces (Zeldis et al., 1972). Cells in culture also produce plasminogen activator (Buonassisi and Venter, 1976; Loskutoff and Edgington, 1977; Loskutoff et al., 1982) and prostaglandin I_2 or prostacyclin (Weksler et al., 1977).

Endothelial cells possess the enzymatic capability to metabolize many vasoactive substances. It is believed that rat lung endothelial cells take up and metabolize serotonin (5-HT)(Junod, 1972; Strum and Junod, 1972). There is also evidence that these cells inactivate norepinephrine via monoamine oxidase and catechol-o-methyl transferase (Hughes et al., 1969). Cultured endothelial cells from rabbit aorta have also been found to synthesize monoamine oxidase (Roth and Venter, 1978). Angiotensin converting enzyme (ACE) has been detected on both pig and rat lung endothelial cells in addition to cultured pig aortic endothelial cells (Ryan et al., 1976).

Many types of receptors have been detected on the endothelial cell surface. Both α-adrenergic and β-adrenergic receptors appear to be present on cultured rabbit aortic endothelial cells (Buonassisi and Venter, 1976). In fact, a catechol-sensitive adenylate cyclase has been

found on the cell membrane and within the cytoplasm of endothelial cells of capillaries isolated from rat epididymal fat (Wagner et al., 1972). Histamine (H2) receptors have also been detected on the luminal aspect of the endothelial cells of mouse diaphragm (Heltianu et al., 1982).

Receptors for both steroid and protein hormones have also been detected. A receptor for the steroid hormone estrogen has been discovered in the cytosol of cultured endothelial cells derived from rabbit aorta (Colburn and Buonassisi, 1978). Likewise, a receptor for the protein hormone insulin has been detected in cultured human endothelial cells (Gimbrone and Alexander, 1977; Bar et al., 1978) and on the endothelia of intact microvessels from bovine cerebral cortex (Frank and Pardridge, 1981) and rat heart (Bar et al., 1982).

Some believe that the receptor sites on the endothelial plasmalemma are composed of proteoglycans and that binding specificity is determined by the types of glycosaminoglycans attached to the protein core of these proteoglycans (Buonassisi and Colburn, 1980). There is evidence that proteoglycans do serve as receptors for lipoprotein lipase on the endothelial cell surface (Wang-Iverson and Brown, 1982).

FUNCTIONAL IMPLICATIONS OF CELL SURFACE CHARGE

Many functions have been postulated for the negative surface charge which is found on both non-endothelial and endothelial cells. In some cell systems, it has been felt that anionic sites play a role in membrane stabilization (Quinton and Philpott, 1973; Katsuyama and Spicer, 1977; DeBruyn and Michelson, 1979). Surface charge has also been shown to affect membrane transport processes (Wojtczak and Nalecz, 1979), cellular aggregation and adhesion (Kemp, 1968) and protein secretion (Glick et al., 1966). Studies have also documented the

necessity of anionic sites in the glomerular capillary for normal glomerular filtration function (Rennke et al., 1975; Kanwar and Farquhar, 1979).

The role of endothelial surface charge in thrombus formation has also been explored. Since blood cells (Sawyer and Pate, 1953a) and most plasma proteins (Sawyer and Pate, 1953a; Blomback and Hanson, 1979) are negatively charged at physiological plasma pH, there is normally a repulsion between the negatively charged blood elements and the capillary wall (Sawyer and Srinivasan, 1972). Indeed, it has been demonstrated that when the vascular intima becomes positive with respect to the adventitia, as can occur with vessel injury, for example, thrombosis is more likely to occur (Sawyer and Pate, 1953a; Sawyer and Pate, 1953b; Schwartz, 1959; Sawyer and Srinivasan, 1972). It is interesting to note that anticoagulant drugs increase repulsive forces between the blood vessel wall and the blood elements, whereas drugs which cause coagulation decrease these repulsive forces (Sawyer and Srinivasan, 1972).

A role for anionic surface charge in capillary transport has also been proposed (M. Simionescu et al., 1981; N. Simionescu, 1981; N. Simionescu et al., 1981; M. Simionescu et al., 1982b; D. Simionescu and M. Simionescu, 1983; N. Simionescu, 1983). It is felt by some that the anionic coated pits are preferentially involved in the transport of cationic proteins (N. Simionescu, 1983) and that the transport of anionic proteins across the capillary wall is facilitated by the non-anionic plasmalemmal vesicles and transendothelial channels (N. Simionescu, 1981; D. Simiomescu and M. Simionescu, 1983). Coated pits are usually involved in adsorptive endocytosis (i.e., the transport of

proteins from the lumen to the lysosome) whereas plasmalemmal vesicles participate in transcytosis (which does not involve this intraendothelial processing). It appears, therefore, that the anionic microdomains allow for the sorting of permeant molecules as a function of charge (N. Simionescu, 1983). These microdomains are discussed in the following section.

ENDOTHELIAL MICRODOMAINS

Recent experiments have demonstrated the existence of anionic microdomains on both the luminal and abluminal surfaces of endothelial cells. The luminal anionic microdomains were first detected in the fenestrated capillaries of mouse pancreas, where it was found that perfused cationized ferritin, a cationic marker for anionic sites (described below) was bound to more than ninety percent of all fenestral diaphragms but to less than ten percent of the diaphragms or membranes of plasmalemmal vesicles and transendothelial channels (N. Simionescu and M. Simionescu, 1978). Further experimentation with fenestrated capillaries of mouse pancreas and jejunal mucosa demonstrated that cationized ferritin bound preferentially to fenestral diaphragms, coated pits and the plasmalemma proper, but was rarely seen in transendothelial channels, plasmalemmal vesicles or bound to their diaphragms (N. Simionescu et al., 1981). The labeling of the plasmalemma was generally uniform, but focal discontinuities in the labeling pattern were noted, and the infundibula leading to the plasmalemmal vesicles were rarely decorated with cationized ferritin (M. Simionescu et al., 1981, N. Simionescu et al., 1981).

The plasmalemma proper of the endothelial cells of liver sinusoids were found to have a patchy distribution of anionic sites. The coated

pits of these cells, however, had both anionic and cationic residues (Ghitescu and Fixman, 1984). Anionic microdomains have also been detected on the luminal aspect of continuous endothelium of murine lung capillaries, detailed below (D. Simionescu and M. Simionescu, 1983), and on the abluminal aspect of fenestrated murine pancreatic and intestinal mucosal capillary endothelium (M. Simionescu et al., 1982b).

The molecular determinants of endothelial cell surface negativity have been described in general. The role of sialic acid in endothelial cell surface negativity is well documented in some microvascular beds (Skutelsky et al., 1975; Skutelsky and Danon, 1976; DeBruyn et al., 1978; DeBruyn and Michelson, 1979; Danon et al., 1980; M. Simionescu et al., 1981; N. Simionescu, 1981) and there is evidence that anionic heparan sulfate is also present on endothelial cells (Buonassisi and Root, 1975; Buonassisi and Colburn, 1983). The partial characterization of specific microdomains has also been reported. The sulfated glycosaminoglycan heparan sulfate is primarily responsible for the anionic charge found on fenestral diaphragms (N. Simionescu et al., 1979; M. Simionescu et al., 1981). The anionic charge of the plasmalemma proper is felt to be due to the presence of both sialoglycoproteins and proteoglycans (M. Simionescu et al., 1981; N. Simionescu, 1981). The anionic sites of coated pits are probably composed of proteoglycans and/or glycoproteins (M. Simionescu et al., 1981) although sialoglycoproteins do not seem to be present at these sites (DeBruyn et al., 1978; M. Simionescu et al., 1981). Acidic glycolipids (gangliosides) do not seem to contribute significantly to any of the anionic microdomains so far identified (M. Simionescu et al., 1981; M. Simionescu et al., 1982b).

Lectins, which bind to specific monosaccharide residues of glycoproteins and glycolipids, have also been used in the characterization of endothelial microdomains. Through the use of specific lectins, the distribution of α-N-acetylgalactosaminyl, β-D-galactosyl, β-N-acetylglucosaminyl, sialyl, α-L-fucosyl, α-D-glucosyl and β-D-mannosyl residues have been determined in murine fenestrated endothelium. These residues were rarely detected on fenestral diaphragms but were found on the plasmalemma proper, coated pits, plasmalemmal vesicles and transendothelial channels. Plasmalemmal vesicles, transendothelial channels and their diaphragms, however, were relatively richer in the neutral monosaccharides β-D-galactose and β-N-acetylglucosamine (M. Simionescu et al., 1980; M. Simionescu et al., 1982a). The endothelial cell, therefore, has differentiated microdomains composed of a preferential distribution of both monosaccharide residues and glycosaminoglycans which define the electrical charge characteristics of these structural elements of the endothelial cells (Figure 2).

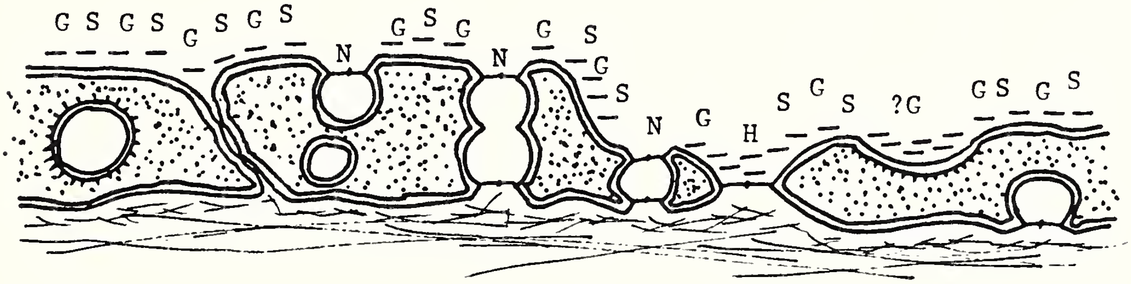

FIGURE 2 Anionic and biochemically defined endothelial microdomains. The other monosaccharide residues described in the text are distributed across the entire luminal aspect of the endothelium, except at the fenestrae and fenestral diaphragms. (-): anionic charge, H: heparan sulfate, S: sialoglycoconjugates, G: glycosaminoglycans including heparan sulfate, N: β-D-galactose and β-N-acetylglucosamine.

CATIONIZED FERRITIN

Cationic probes have been used in conjunction with electron microscopy in the study of cell surface negativity. In the past, colloidal iron hydroxide was often used for this purpose, but was of limited utility because tissues had to be prefixed and studied at a pH of 1.8 (Gasic et al., 1968; Weiss and Zeigel, 1971; Skutelsky et al., 1975). The introduction of cationized ferritin, however, has allowed for the study of unfixed cells at physiological pH (Danon et al., 1970; Danon et al., 1972; Subjeck and Weiss, 1975) and experiments using this probe have shown that the amount of cationized ferritin bound is correlated with the degree of cell surface negativity (Danon et al., 1972).

MOLECULAR STRUCTURE

Horse spleen ferritin, usually referred to as native ferritin, is the molecule from which cationized ferritin is derived. This roughly spherical molecule (diameter: approximately 12 nm) is composed of an inner iron oxide hydrate core, with an approximate diameter of 7.5 nm and an outer protein shell, called apoferritin (Harrison, 1963; Hoffman and Harrison, 1963) (Figure 3). Apoferritin is made up of twenty identical 24,000 dalton subunits, which are oriented at the vertices of a pentagonal dodecahedron, to give the molecule its pseudo-icosahedral symmetry (Harrison et al., 1962; Hofmann and Harrison, 1963; Haggis, 1965). The electron dense iron core is uniform in density and can be visualized with the electron microscope without staining (Haggis et al., 1965).

Danon et al. found that native ferritin, which has a pI of 4.5 and

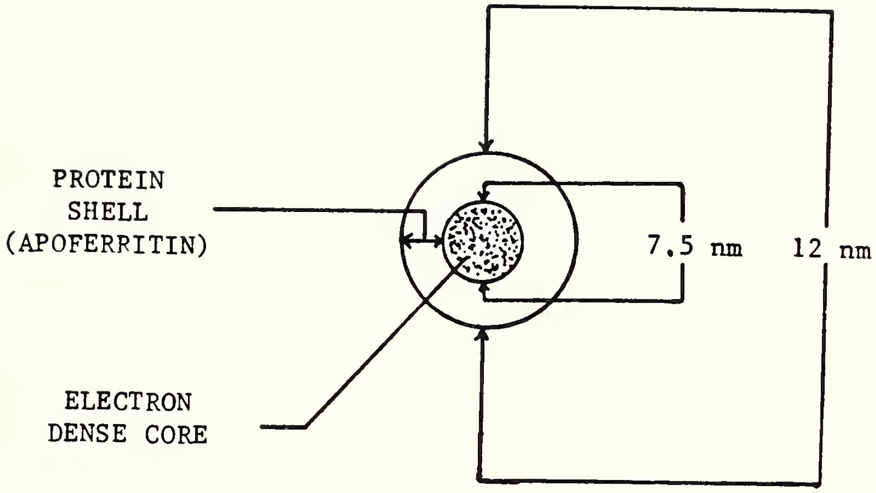

FIGURE 3 Structure of native ferritin. The dimensions given are for ferritin in the wet state; air dried crystals are slightly smaller. The molecular weight of native ferritin (core plus apoferritin) is 750,000 daltons.

therefore a negative charge at physiological pH, could be cationized through an alteration of the molecule's carboxyl groups (Danon et al., 1972). The method they used, and which is still used presently, is outlined in Figure 4. The carboxyl groups of native ferritin are activated with a carbodiimide and then reacted with a nucleophile. The modification of these negatively charged carboxyl groups with positively charged molecules results in the net addition of two positive charges for every carboxyl group changed. Native ferritin can be modified in this manner to have a pI of 8.4 or greater and therefore be positively charged at physiological pH. As can be determined by electron microscopy, the synthesized cationized ferritin does not differ morphologically from native ferritin (Danon et al., 1972).

USE IN THE STUDY OF CONTINUOUS ENDOTHELIUM

The earliest report in the literature in which cationized ferritin

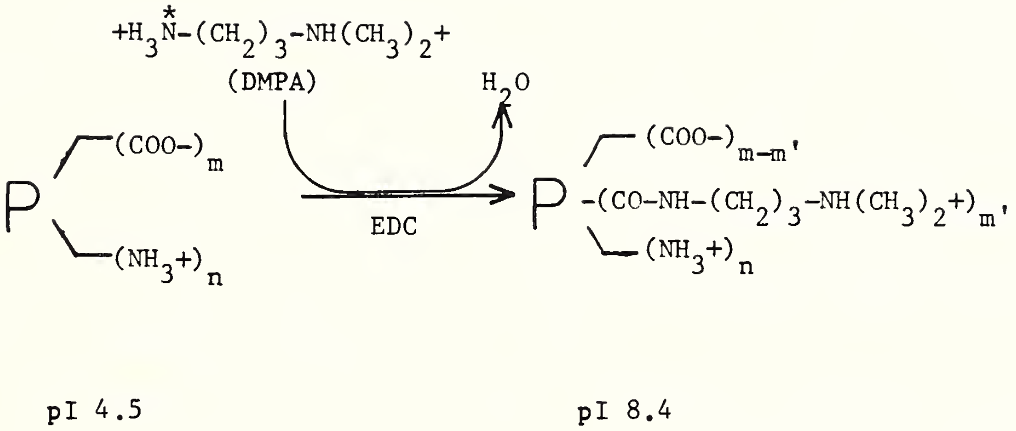
$+H_3\overset{*}{N}-(CH_2)_3-NH(CH_3)_2+$
(DMPA)
H_2O
P
$(COO-)_m$
$(NH_3+)_n$
EDC
P
$(COO-)_{m-m'}$
$-(CO-NH-(CH_2)_3-NH(CH_3)_2+)_{m'}$
$(NH_3+)_n$
pI 4.5
pI 8.4

FIGURE 4 Synthesis of cationized ferritin from native ferritin. P: protein shell of ferritin (apoferritin), m: number of carboxyl groups, m': number of carboxyl groups modified, n: number of amino groups, EDC: 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride, DMPA: N,N-dimethyl-1,3-propanediamide, *: reactive site.

was used in the characterization of the anionic nature of the cell surface of continuous endothelium was based on experimentation done with rabbit ear microvessels (Danon, 1970). In this and other experiments on these microvessels, cationized ferritin was shown to uniformly label the entire luminal membrane, without evidence of preferential binding (Danon, 1970; Danon et al., 1972). Frog mesenteric capillaries, which have a continuous endothelium (Renkin, 1977), also bound cationized ferritin uniformly when perfused with this probe in vivo (Clough, 1981; Clough, 1982; Turner et al., 1983).

Cationized ferritin, when incubated with rat aorta, was found to be evenly distributed on the luminal surface of these continuous endothelial cells (Laver-Rudich et al., 1978; Danon et al., 1980). The continuous endothelium of guinea pig aorta (Skutelsky et al., 1975;

Danon and Skutelsky, 1976; Skutelsky and Danon, 1976) and vena cava (Skutelsky et al., 1975; Skutelsky and Danon, 1976), and rabbit aorta (Skutelsky et al., 1975; Danon and Skutelsky, 1976) and vena cava (Skutelsky et al., 1975) also exhibited uniform labeling of cationized ferritin, without any evidence of microdomains, when studied. Like the endothelial cells of these macrovessels, cultured human umbilical vein endothelial cells, both primary and SV40-transformed, bound cationized ferritin uniformly (Pelikan et al., 1979).

There are two examples in the literature, however, in which cationized ferritin was found to bind in a preferential manner to particular luminal components of continuous endothelial cells. In one study the continuous endothelia lining myeloid sinuses was examined. A uniform layer of cationized ferritin was observed on these cells, without evidence of microdomains, but at the sites where the newly formed blood cells traverse the endothelium to enter into the circulation, marked accumulations of cationized ferritin were noted (DeBruyn and Michelson, 1981). In the other study, lung capillary endothelium was probed. It was found that in the avesicular area of the endothelial cell, no significant amount of cationized ferritin was bound, but that in the vesicular area, cationized ferritin labeled the luminal plasmalemma proper but did not decorate the plasmalemmal vesicles or transendothelial channels (D. Simionescu and M. Simionescu, 1983).

MICROVESSELS OF MURINE DIAPHRAGM

The arterioles, capillaries and venules of the mouse diaphragm, like other skeletal muscle microvessels, are provided with a continuous endothelium. These microvessels have been studied with the aid of the electron microscope and many of their morphological characteristics have been described. The bipolar microvascular fields present in murine diaphragm have aided in this characterization (N. Simionescu et al., 1978a; N. Simionescu et al., 1978b).

In the bipolar microvascular fields, the capillaries are supplied at one end by arterioles and drained at the other end by venules. These elements of the microcirculation are arranged linearly in the diaphragmatic tissue and are easier to detect in the dorso-lateral section of each hemidiaphragm. As the result of the systematic electron microscopic study of the segments along these bipolar microvascular fields, criteria are available which aid in the identification of microvessel segments (eg., arteriole vs. venule).

Arterioles were found to have an inner diameter of between 20 and 50 μm, a discontinuous internal elastic lamina and a continuous, usually single layered, muscle tunic. Metarterioles are similar to arterioles, but have a slightly smaller inner diameter (10 to 20 μm) and no internal elastic lamina. Capillaries have an inner diameter of about 4.75 μm in their smallest transverse dimension. Pericytic venules are characterized by an inner diameter of 12 to 25 μm, a more attenuated endothelium and a discontinuous layer of pericytes. Muscular venules have a large inner diameter (between 20 and 40 μm) and a layer of smooth muscle in their relatively thin wall (N. Simionescu, et al., 1978a).

Differences in the frequency and character of some endothelial

structural elements were also noted along the different segments of the bipolar microvascular fields. The density of plasmalemmal vesicles was found to be highest in the capillaries, followed by pericytic venules, muscular venules and finally arterioles. Transendothelial channels were also more commonly found in the capillary segment of the bipolar microvascular fields. The character of the intercellular junctions differed between segments of this microvasculature. All junctions were impermeable to a 2 nm tracer, except some of those at the level of the pericytic venules, where 25 to 30 percent of the intercellular junctions were open to a gap of 3 to 6 nm (N. Simionescu et al., 1978b).

These microvessels are the subject of this study. Cationized ferritin was used to probe the distribution of anionic sites on the luminal aspect of the murine microvasculature with the intent of determining whether anionic microdomains exist in the continuous endothelium of skeletal muscle.

CURRENT RESEARCH

MATERIALS AND METHODS

Animals

Two groups of Swiss albino mice were used in this study. The first group was composed of eight young male mice, between six and eight weeks old, and weighing approximately 20 grams. The second, more heterogeneous group was composed of seven male and three female mice, approximately one year old, and weighing between 30 and 47 grams.

Experimental protocol

Each animal was anesthetized with one milliliter per 100 grams body weight of 5% chloral hydrate solution, injected intra-peritoneally. This was supplemented with inhaled ether as necessary. A laparotomy was performed, cutting along the linea alba to minimize bleeding, and the viscera were retracted to expose the abdominal aorta and vena cava (Cook, 1965). After these vessels were adequately visualized with the aid of a dissecting microscope, a small gauge needle was advanced into the aorta, and a hole was made in the wall of the vena cava abdominalis.

In order to free the vasculature of blood, retrograde perfusion was initiated through the aorta with Dulbecco's phosphate-buffered saline (DPBS), pH 7.2 to 7.4, supplemented with 14 mM glucose and 100 mg per liter of calcium chloride. This solution was prewarmed to 37°C and gassed with 95% oxygen and 5% carbon dioxide. The animal was perfused for five minutes at a rate of 3 ml per minute.

After this initial perfusion of the vasculature, 2.5 ml per 100 gram body weight of a solution of 2 mg per ml cationized ferritin (in DPBS) was slowly injected through the aorta. Both the aorta and vena cava were simultaneously ligated at this point. After a period of

three, five, ten or twenty minutes, the ligature around the aorta and vena cava was released, and the vasculature was again washed with the supplemented DPBS solution described above for three minutes at a rate of 3 ml per minute. The tissue was then fixed in situ for ten minutes with the retrograde perfusion of 7 ml of 5% formaldehyde (prepared freshly from paraformaldehyde) and 3% glutaraldehyde dissolved in a 0.1 M HCl-Na cacodylate buffer (pH 7.2 to 7.4). Small cotton balls soaked with this fixative were also placed on the abdominal surface of the diaphragm during this initial phase of fixation.

Processing of tissue for electron microscopy

After fixation in situ, the diaphragm was carefully removed from the carcass of the animal, placed in a petri dish and covered with the fixative solution described above. The immersed diaphragm was placed under a dissecting microscope and small pieces of tissue were excised in the area of the bipolar microvascular fields with a razor blade. These specimens were placed in vials containing the same paraformaldehyde and glutaraldehyde fixative for 80 additional minutes.

The tissue was rinsed twice with 0.1 M HCl-Na cacodylate buffer (pH 7.4) and then post-fixed with 1% OsO_4 in a 0.1 M HCl-Na cacodylate buffer for 90 minutes at 4°C. Following this final fixation, the tissue was rinsed three times (five minutes per rinse) with Michaelis buffer and then stained in block with 0.5% uranyl acetate in Michaelis buffer (pH 5.5) for thirty minutes.

After washing with double distilled water, dehydration was accomplished through the incubation of the tissue in graded ethanol solutions (70%, 95% and 100% ethanol). The tissue was dehydrated with each of the lower concentraton ethanol solutions for five minutes and then with the pure (100%) ethanol solution for 20 minutes total. The

tissue was further treated with propylene oxide for thirty minutes and then embedded in Epon 812.

Electron microscopy

Thin (60 nm) sections cut on either a Sorvall or Reichert ultramicrotome were placed on copper grids, previously coated with formvar and carbon. The sections were stained with uranyl acetate for one minute followed by lead citrate for three minutes. The preparations were then examined and random microvascular profiles were photomicrographed using a Philips 201 or 301 electron microscope operated at 80 kV.

Morphometry

For each micrograph, the fraction of the luminal perimeter labeled with cationized ferritin was determined with the use of a planimeter. From this data, the mean and standard deviation of the mean of the percentage of the luminal plasmalemma labeled were calculated for young and old animals in sequential segments of the microvasculature: arterioles, capillaries, and venules. The plasmalemmal vesicles open to the blood front or located within 10 nm from it were also counted for each vessel type examined. It was assumed that vesicles within 10 nm of the luminal surface may be actually open to the blood front in another plane of section. The percentage of these vesicles labeled with cationized ferritin, either on their membrane or within the vesicle, was determined.

A total of 454 electron micrographs were used in this study. The aggregate lengths of plasmalemma examined were 265.0 μm, 679.4 μm, and 902.3 μm for arterioles, capillaries and venules, respectively. A total of 1744 plasmalemmal vesicles were counted. For a more specific

description of the sampling, refer to Tables 1 and 2.

Statistical analysis

The data was analyzed using a two-tailed Student's t-test (Snedecor and Cochran, 1967). P values less than 0.05 implied statistical significance.

The sources of the chemicals used, and the composition of buffers and solutions employed are given in Appendix A and Appendix B, respectively.

RESULTS

In all microvascular segments studied, cationized ferritin was generally found to bind to the luminal plasmalemma of the endothelium in patches composed of one to three layers of particles (Figure 5 through 10). These patches were of variable size and were separated by variable lengths of unlabeled plasmalemma. The patchy binding extended into the luminal part of the interendothelial spaces, but was never found beyond the intercellular junctions (Figure 11). Cationized ferritin was never detected on the abluminal plasmalemma or in the pericapillary space. Binding did not vary with the different incubation times (three to twenty minutes) or with the sex of the animal examined. The pattern of binding was also similar for both the perikaryon and the attenuated segment of the endothelial cells.

In the diaphragm microvessels of young mice, the percentage of the endothelial plasmalemma of arterioles and venules labeled with cationized ferritin was comparable ($P>0.5$), and both of these values were approximately 20% greater than the percentage of binding found in young mouse capillary endothelium ($P<0.005$, $P<0.001$ respectively). This

same relationship was found between the three segments of the old mouse diaphragm microvasculature. The percentage of arteriolar and venular plasmalemma labeled was comparable ($P>0.5$) but was 10% greater than the percentage of capillary endothelium labeled ($P<0.01$, $P<0.005$ respectively).

Differences were also found in the extent of plasmalemmal labeling of young and old microvessel endothelia. The percent of the luminal plasmalemma labeled in old mouse arterioles was 13% less than that found in young mouse arterioles ($P<0.025$) and the percent of the plasmalemma labeled in old mouse venules was 10% less than that found in young mouse venules ($P<0.005$). There was no statistically significant difference in the percent of labeling when young and old mouse capillaries were compared ($P>0.2$)(Table 1).

The majority of plasmalemmal vesicles open to the lumen or within 10 nm from the lumen were not labeled with cationized ferritin. The percentage of plasmalemmal vesicles labeled was very similar (between 22 and 27 percent) for the arterioles, capillaries and venules of both young and old mouse diaphragm (Table 2). When labeled, the vesicles were decorated with only a few cationized ferritin molecules (Figures 8, 12). The stomatal diaphragms of the plasmalemmal vesicles, when present, typically did not bind the cationized ferritin (Figure 13).

Coated pits and transendothelial channels were rarely detected in the micrographs examined. Those coated pits observed, however, were heavily labeled with cationized ferritin (Figure 12) whereas channels were not decorated.

TABLE 1

Percentage of Luminal Plasmalemma Labeled With Cationized Ferritin (pI 8.4)

	arterioles	capillaries	venules
Young Animals:			
percentage of luminal plasmalemma labeled	54 ± 11	45 ± 10	53 ± 12
number of micrographs examined	16	62	119
aggregate perimeter examined, micrometers	66.2	304.6	444.5
Old Animals:			
percentage of luminal plasmalemma labeled	47 ± 8	43 ± 9	48 ± 13
number of micrographs examined	56	74	127
aggregate perimeter examined, micrometers	198.2	374.9	457.8

±SD, Standard deviation of the mean

TABLE 2

PERCENTAGE OF LUMINAL PLASMALEMMAL VESICLES* LABELED WITH CATIONIZED FERRITIN (pI 8.4)

	arterioles	capillaries	venules
Young Animals:			
percentage of plasmalemmal vesicles labeled	22	22	26
total number of vesicles counted	76	422	447
Old Animals:			
percentage of plasmalemmal vesicles labeled	27	24	23
total number of vesicles counted	116	379	304

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*vesicle population consists of plasmalemmal vesicles open to the lumen or within ten nanometers of the lumen, as described in the text.

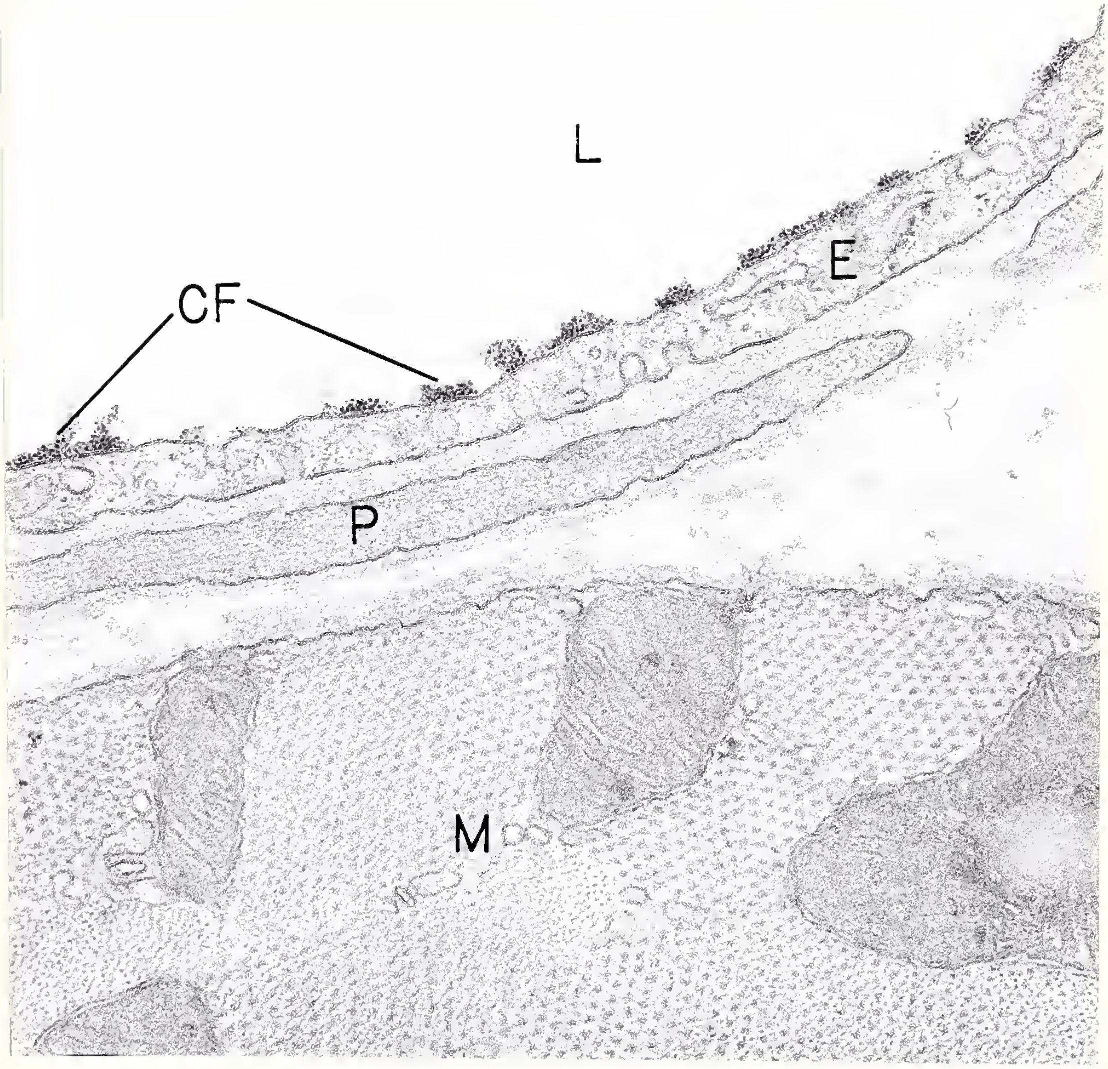

FIGURE 5 Venule from the diaphragm of a young mouse, X63,000. Note the patchy labeling of the luminal plasmalemma with cationized ferritin and the absence of plasmalemmal vesicle labeling. L: lumen; E: endothelial cell; P: pericyte; M: diaphragm muscle; CF: cationized ferritin particles.

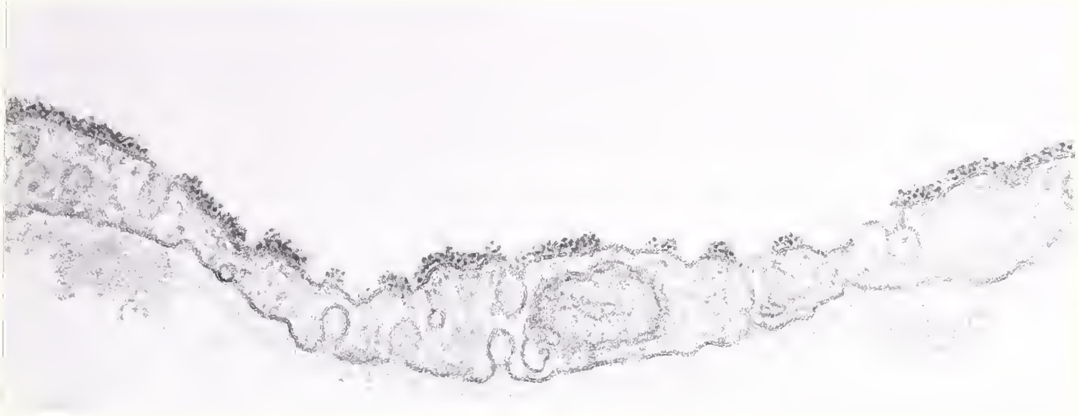

FIGURE 6 Capillary from the diaphragm of a young mouse, X73,000. In most labeled areas cationized ferritin decorates the luminal plasmalemma as a double layer of particles.

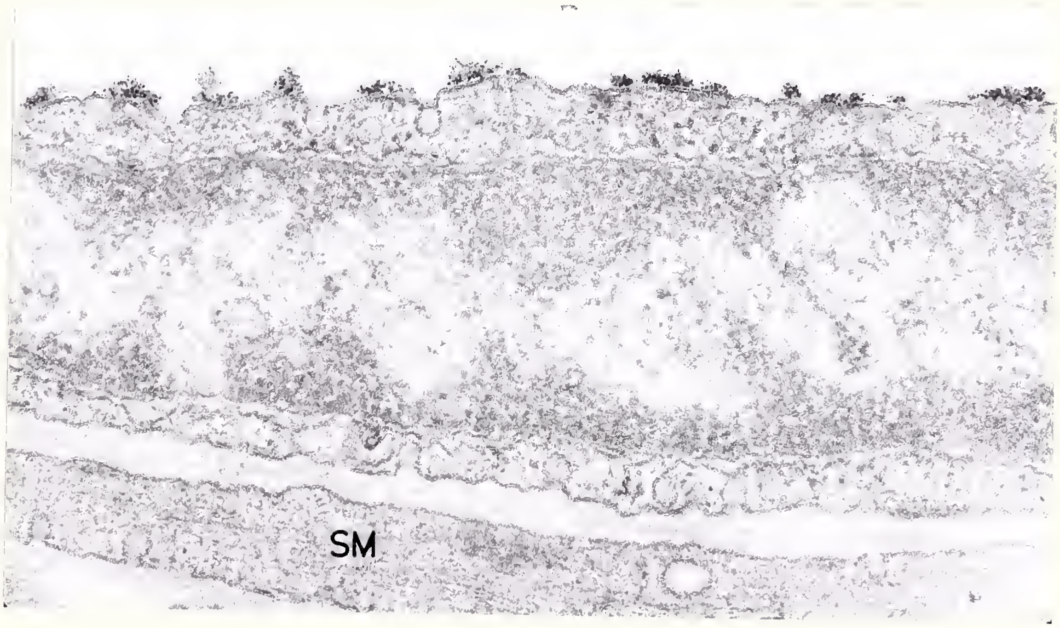

FIGURE 7 Arteriole from the diaphragm of a young mouse, X59,000. SM: smooth muscle cell of arteriole wall.

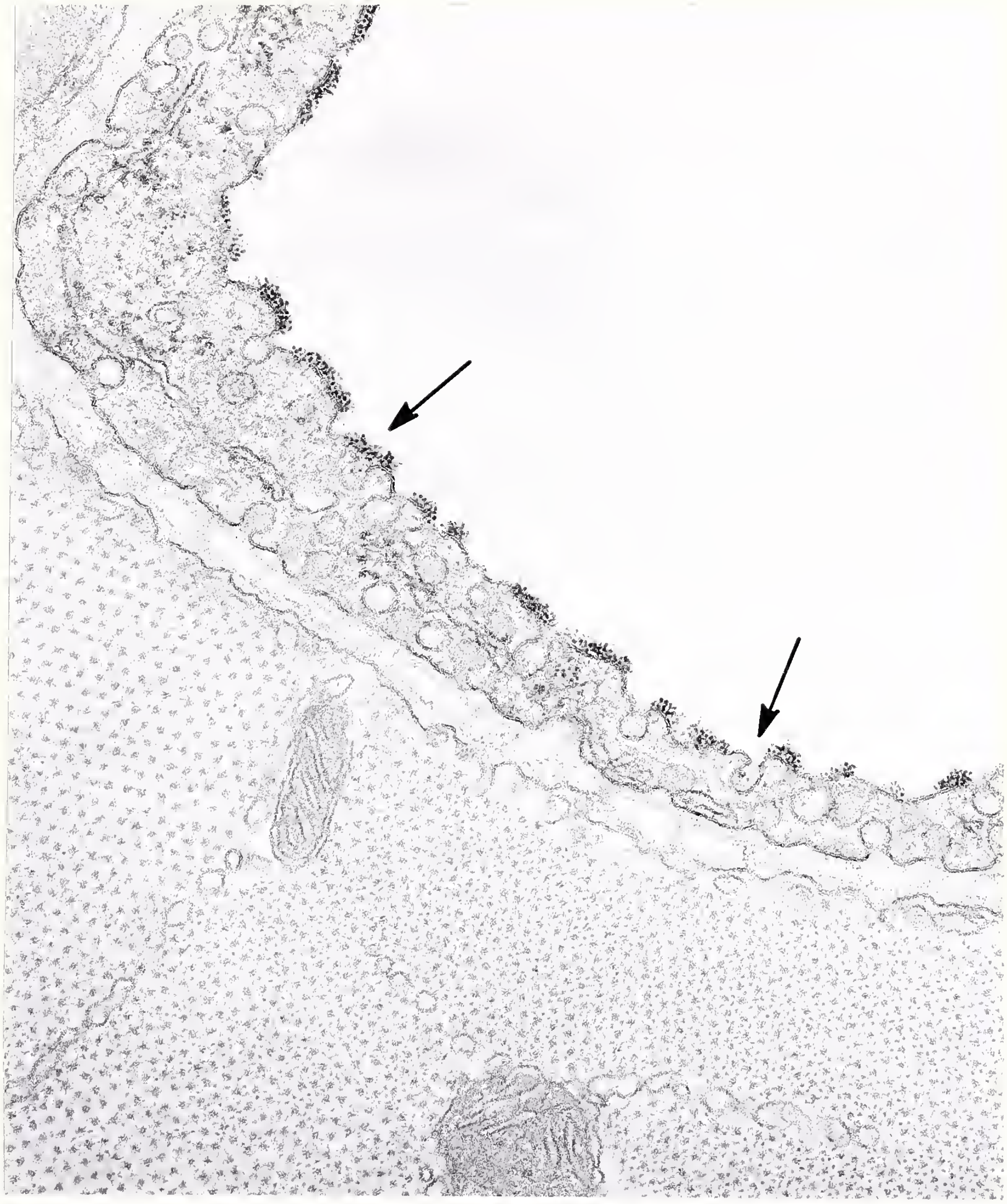

FIGURE 8 Capillary from the diaphragm of a young mouse, X63,000. Patchy distribution of cationized ferritin on the plasmalemma. Most plasmalemmal vesicles are not labeled. The plasmalemmal vesicles labeled (arrow) are decorated with only a few cationized ferritin molecules.

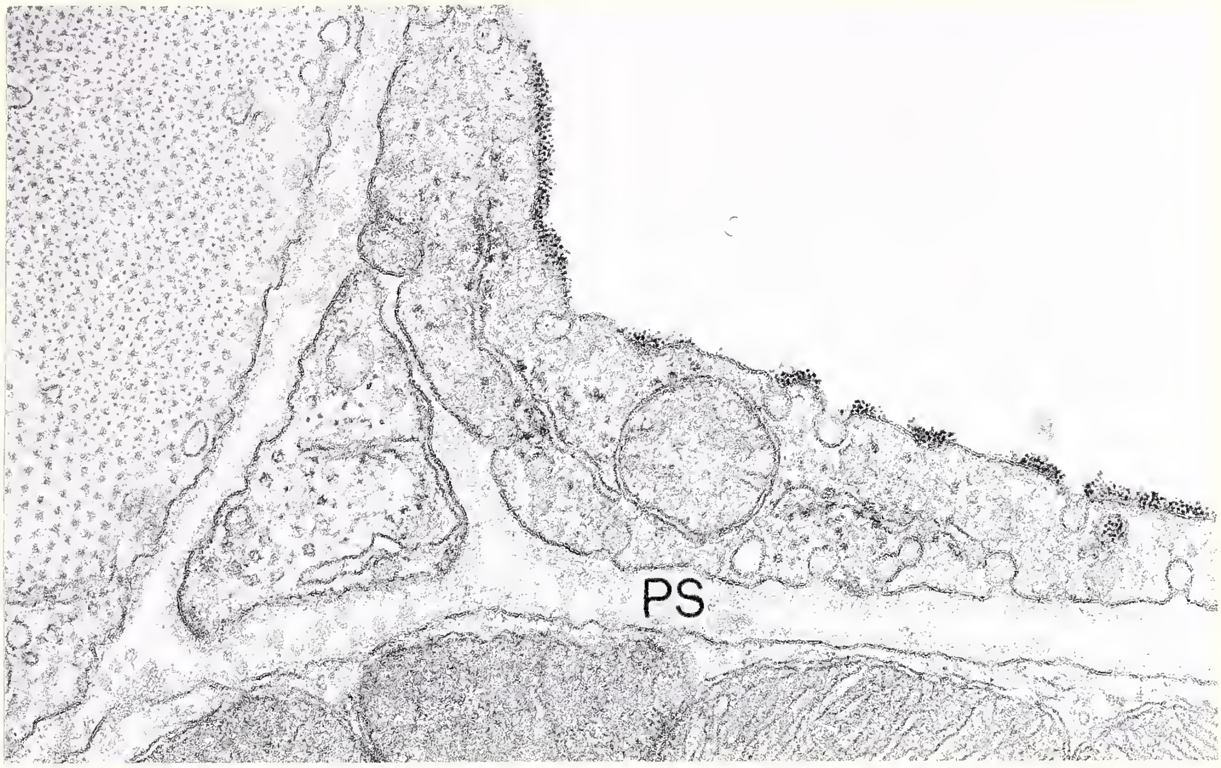

FIGURE 9 Capillary from the diaphragm of a young mouse, X63,000. Cationized ferritin is not detected in the pericapillary space. PS: pericapillary space.

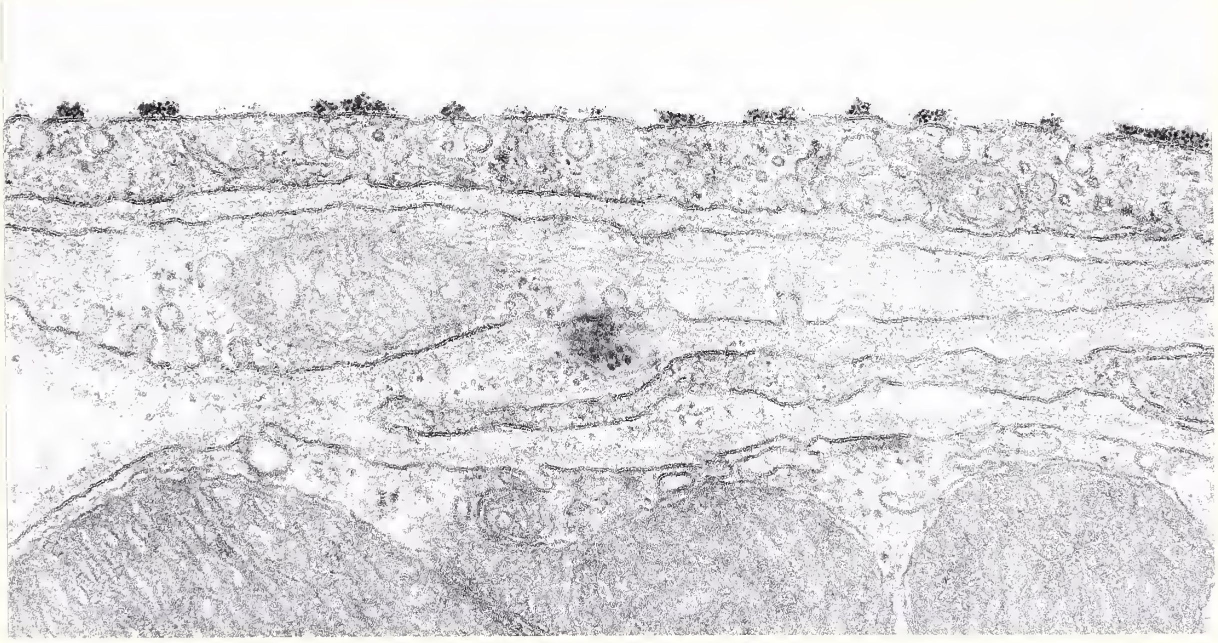

FIGURE 10 Venule from the diaphragm of an old mouse, X65,000.

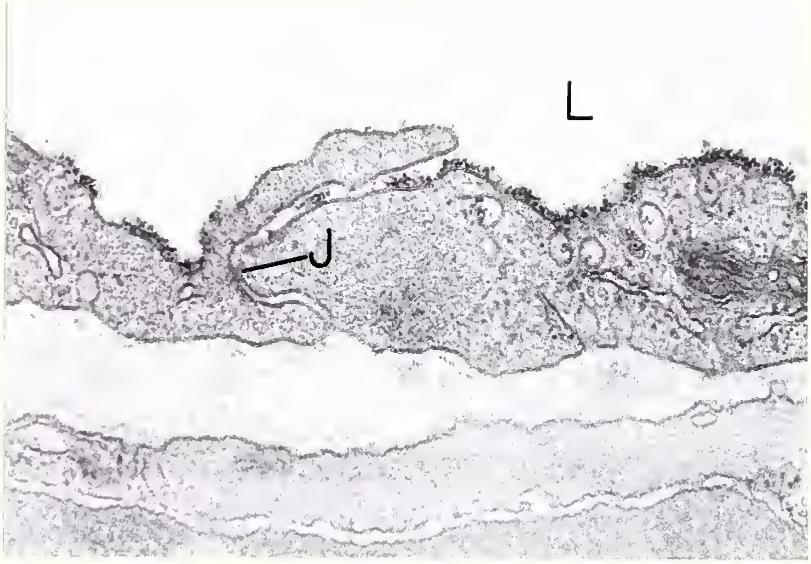

FIGURE 11 Venule from the diaphragm of a young mouse, X59,000. Cationized ferritin decorates the luminal plasmalemma but does not go beyond the intercellular junction. The high percentage of luminal plasmalemmal vesicles labeled with cationized ferritin is not characteristic. L: lumen; J: intercellular junction.

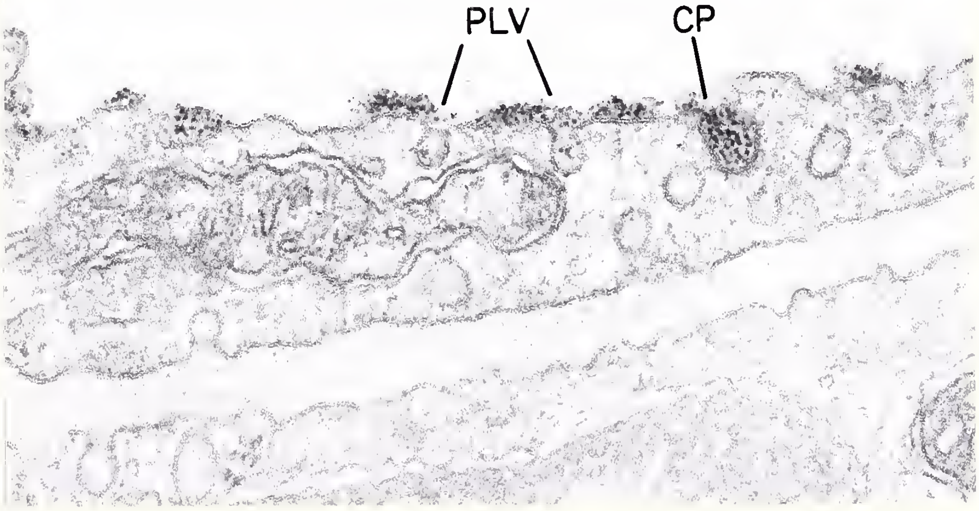

FIGURE 12 Venule from the diaphragm of a young mouse, X100,000. The coated pit is heavily labeled with cationized ferritin particles, whereas the plasmalemmal vesicles are decorated with only a few particles. CP: coated pit; PLV: plasmalemmal vesicle.

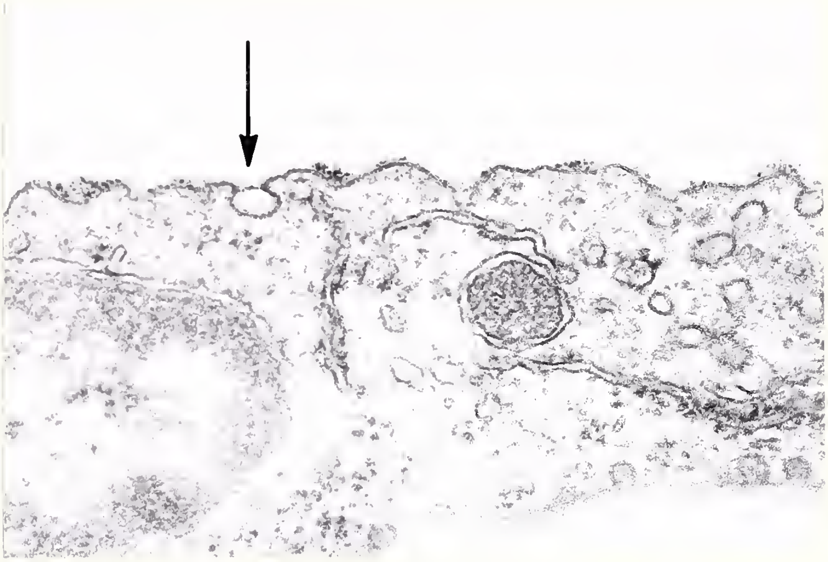

FIGURE 13 Venular endothelial cell from the diaphragm of an old mouse, X59,000. The plasmalemmal vesicle and its diaphragm (arrow) are not labeled by the cationized ferritin.

DISCUSSION

The fine distribution of anionic sites on the luminal aspect of the continuous endothelium of mouse diaphragm microvessels was found to be similar to the distribution of anionic sites described in the vesicular areas of lung capillary endothelium (D. Simionescu and M. Simionescu, 1983) and the non-fenestrated areas of visceral capillary endothelium (N. Simionescu and M. Simionescu, 1978; N. Simionescu et al., 1981). The plasmalemma was heavily labeled with cationized ferritin, whereas the majority of plasmalemmal vesicles were free of cationized ferritin decoration.

The distribution of perfused cationized ferritin found on the luminal plasmalemma of the endothelia of murine diaphragm microvessel was more patchy in nature than the distribution described for either lung or visceral vascular endothelia. There are reports in the literature in which cationized ferritin was found to bind to cells in a patchy manner if the cells were incubated with cationized ferritin before fixation, but to bind in a continuous manner if fixation preceded labeling (Marikovsky et al., 1974; Subjeck and Weiss, 1975; Skutelsky and Danon, 1976; Moller and Chang, 1978a; Moller and Chang, 1978b; King, 1979; Pelikan et al., 1979; Danon et al., 1980; Marikovsky and Weinstein, 1981). The conclusion drawn fron these studies was that cationized ferritin could cause the lateral migration of anionic components within the plasmalemma. It is also possible, however, that fixation itself may have modified the actual distribution of anionic charge. With respect to in situ vascular labeling experiments, incomplete removal of plasma proteins (due to the inadequate washing of the vasculature prior to cationized ferritin exposure) will also cause

continuous labeling. The patchy nature of cationized ferritin binding found in this study does not appear to be artifactual. Further experiments on these microvessels involving initial fixation, in situ, followed by cationized ferritin perfusion (after quenching the free aldehyde groups with glycine) yielded the same discontinuous binding pattern (M. Simionescu et al., in press).

The percentage of plasmalemmal vesicles labeled by cationized ferritin in this study was higher than the percentage of vesicles labeled in visceral vascular endothelium (N. Simionescu and M. Simionescu, 1978). The majority (approximately 75%) of plasmalemmal vesicles were free of cationized ferritin decoration, however, and those which were labeled with cationized ferritin were only decorated with a few particles. There were differences in the labeling pattern of plasmalemmal vesicles compared to the plasmalemma proper. Specifically, the content, more often than the membrane of labeled plasmalemmal vesicles was decorated with cationized ferritin.

The plasmalemmal vesicles of this continuous endothelium could serve as the preferential carrier for anionic and neutral plasma proteins. Transport of proteins may be facilitated by the local absence of negative surface charge, due to a reduction in the electrostatic repulsion between the endothelial cell of the microvessel wall and the transported protein. This supposition is supported by evidence that native ferritin (pI 4.6) has access to a large percentage of plasmalemmal vesicles in the continuous endothelium of mouse diaphragm (Bruns and Palade, 1968b) and frog mesenteric capillaries (Loudon et al., 1979).

In this study, no attempt was made to characterize the nature of the detected endothelial microdomains. Further experiments on the

microvessels of young mouse diaphragm, however, have shown that the anionic sites on the luminal aspect of this endothelium are composed of sialoglycocproteins and proteoglycans, but not sialoglycolipids (M. Simionescu et al., in press). Furthermore, sulfated proteoglycans do not appear to be major contributors to the anionic sites found on the luminal endothelium of these microvessels. Presently, there is not enough information concerning the luminal distribution of endothelial surface enzymes and receptors to correlate their position on the endothelial plasmalemma with the location of these specific microdomains.

There appears to be variation in the distribution of luminal anionic charge of the continuous endothelium of large vessels compared to the distribution found on the continuous endothelium of microvessels. No anionic microdomains were detected on the luminal aspect of the continuous endothelium of aorta (Skutelsky et al., 1975; Danon and Skutelsky, 1976; Skutelsky and Danon, 1976; Laver-Rudich et al., 1978; Danon et al., 1980), vena cava (Skutelsky et al., 1975; Skutelsky and Danon, 1976) and umbilical vein (Pelikan et al., 1979). Anionic microdomains were detected, however, on the luminal aspect of the continuous endothelial cells of lung (D. Simionescu and M. Simionescu, 1983) and diaphragm microvessels. Anionic microdomains were not detected on the luminal aspect of frog mesenteric microvessels (Clough, 1981; Clough, 1982; Turner et al., 1983), but very high concentrations of cationized ferritin (up to 25 times higher than the concentrations used in this study) were used in these experiments. Cationized ferritin with a high pI (>9) was also used in these studies. Similarly, high (50 mg per ml) concentrations of cationized ferritin (of unspecified pI)

were used in experiments with rabbit ear microvessels (Danon et al., 1972); microdomains also were not detected on the luminal aspect of the endothelial cells of these microvessels. The fine distribution of anionic sites may be masked by either the use of high concentrations of cationized ferritin or by the use of high pI cationized ferritin solutions. Excessive concentrations of cationized ferritin could cause clumping of this ligand. Likewise, cationized ferritin of high pI may bind to sites which have relatively less negative surface charge, thus decreasing the resolution of this technique along with the ability to detect microdomains. Further experiments are needed to determine whether anionic microdomains can be detected on the luminal endothelium of these and other microvesels with the use of lower concentration cationized ferritin solutions of well defined pIs.

The general cationized ferritin labeling pattern found on the luminal plasmalemma of the endothelium was the same for each of the microvascular segments examined (arterioles, capillaries, venules). The percentage of plasmalemmal vesicles labeled in each segment of the microvasculature was comparable, yet some segmental variation was detected in the extent of plasmalemmal labeling. A slightly smaller percentage of the luminal perimeter of capillary endothelium was labeled than arteriolar or venular endothelium. The significance of this difference is not apparent.

The general pattern of cationized ferritin binding on the luminal plasmalemma was also similar for the endothelial cells of the diaphragm microvessels of both young and old mice. There was a statistically significant decrease, however, in the percentage of the luminal plasmalemma labeled with perfused cationized ferritin in the arterioles and venules of old animals compared to young animals. Since endothelial

cell surface negativity is known to be important in normal vascular homeostasis and the prevention of thrombus formation, this decrease in the distribution of negative charge seen in old animals may be causally related to the development of atherosclerosis and microthrombosis seen more frequently with aging.

Differences in the intensity of endothelial labeling should also be considered, as the density of cationized ferritin binding correlates with the degree of cell surface negativity. Further work on mouse diaphragm microvessels has shown a decrease in the intensity of cationized ferritin binding to the luminal plasmalemma of venular endothelium of old animals when compared to young animals (M. Simionescu et al., in press). The age of the old mice used in those experiments was 23 months, which is greater than both the age of the old mice used in this study and the average life expectancy (20 months) of Swiss mice (Storer, 1967). The observations of M. Simionescu et al. do lend further support to a causal relationship between the age associated increase in thrombogenesis and a decrease in cell surface negativity secondary to aging.

APPENDIX A

Source of Chemicals

cacodylic acid: Sigma, St. Louis, Missouri

calcium chloride: Merck, Darmstadt, West Germany

cationized ferritin: Miles-Yeda Ltd., Rehovot, Israel

chloral hydrate: Riedel-de Haen AG, Hannover, West Germany

Dulbecco's phosphate-buffered saline (DPBS): Gibco Co., New York

epon 812: JBS, Canada

ethanol: Reactivul, Bucharest, Romania

ether: Reactivul, Bucharest, Romania

formvar: Serva, Heidelberg, West Germany

glucose: Merck, Darmstadt, West Germany

glutaraldehyde: JBS, Canada

hydrochloric acid: Reactivul, Bucharest, Romania

lead citrate: Merck, Darmstadt, West Germany

osmium: Merck, Darmstadt, West Germany

paraformaldehyde: Fischer, Fair Lawn, New Jersey

propylene oxide: Reactivul, Bucharest, Romania

sodium acetate: Merck, Darmstadt, West Germany

sodium barbiturate: Merck, Darmstadt, West Germany

uranyl acetate: Merch, Darmstadt, West Germany

APPENDIX B

Solution Compositions

Veronal Acetate Buffer (500 ml)

9.714 grams of sodium acetate

14.714 grams of sodium barbiturate

double distilled water to bring final volume to 500 ml

Michaelis Buffer (100 ml)

20 ml of veronal acetate buffer

20 ml of 0.1 N HCl

double distilled water to bring final volume to 100 ml

adjust pH to 5.5

0.5% Uranyl Acetate Solution (100 ml)

20 ml of veronal acetate buffer

28 ml of 0.1N HCl

0.5 grams of uranyl acetate

double distilled water to bring final volume to 100 ml

adjust pH to 5.5

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